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<b>(54) Title:</b> TRANSGENIC ANIMALS HAVING A DISRUPTED ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE AND METHODS OF USE  <b>(57) Abstract</b>  This invention relates to transgenic non-human animals comprising a disrupted endothelial nitric oxide synthase gene. These animals exhibit abnormal wound-healing properties and hypertension. This invention also relates to methods of using the transgenic animals to screen for compounds having anti-hypertensive and wound-healing activities. Moreover, this invention also relates to methods of treating a patient suffering from hypertension and wound-healing abnormalities with the compounds identified using the transgenic animals, and methods of making the transgenic animals. A method of treating a wound using nitroglycerin is also provided.		

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TRANSGENIC ANIMALS HAVING A DISRUPTED ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE  
AND METHODS OF USE

5

***Background of the Invention***

***Statement as to Rights to Inventions Made Under  
Federally-Sponsored Research and Development***

10 Part of the work performed during the development of this invention was  
supported by U.S. Government funds. The U.S. Government may have certain  
rights in this invention.

***Field of the Invention***

15 This invention relates to transgenic non-human animals comprising a  
disrupted endothelial nitric oxide synthase gene. This invention also relates to  
methods of using these transgenic animals to screen compounds for anti-  
hypertensive and wound-healing activity, methods of treating a patient suffering  
from hypertension or wound healing abnormalities, methods of making the  
transgenic animals, and cell lines comprising a disrupted eNOS gene.

***Related Art***

20 Nitric oxide is an important messenger molecule produced by endothelial  
cells, neurons, macrophages, and other tissues. Marletta (1989); Moncada  
(1991); Nathan (1992); Snyder (1992); and Dawson *et al.* (1992). Since nitric  
oxide is a gas with no known storage mechanism, it diffuses freely across  
membranes and is extremely labile. Nitric oxide has a biological half-life on the  
25 order of seconds.

Nitric oxide exhibits several biochemical activities. This compound can bind to and activate soluble guanyl cyclase, resulting in increased cGMP levels. Nitric oxide also modifies a cysteine residue in glyceraldehyde-3-phosphate dehydrogenase by adenosine diphosphate ribosylation, Zhang & Snyder (1992), Katz *et al.* (1992), and Dimmeler *et al.* (1992), or S-nitrosylation via NAD interactions, McDonald & Moss (1993). Nitric oxide also binds to a variety of iron- and sulphur-containing proteins, Marletta (1993), and may have other modes of action as well.

Nitric oxide formation is catalyzed by the nitric oxide synthase enzymes (NOS). These enzymes act by producing nitric oxide from the terminal guanidino nitrogen of arginine, with the stoichiometric production of citrulline. There are several NOS isoforms encoded by separate genes. Marletta (1993), and Lowenstein & Snyder (1992). The various NOS isoforms are about 50-60% homologous overall. Some forms of NOS is found in most tissues. The different NOS isoforms: neuronal NOS (nNOS), macrophage NOS (mNOS), and endothelial NOS (eNOS), are now known as type I NOS, type II NOS and type III NOS, respectively. The ubiquitous presence of blood vessels and nerves means that the endothelial and neuronal isoforms may be present in most tissues. The sequence of these isoforms have been published or are available in Genbank under the following accession numbers:

Gene:	Species:			
	Man	Rat	Mouse	Cow
Neuronal (type I)	U17327	X59949	D14552	
	D16408			
	L02881			
Macrophage (type II)	L09210	D14051	M87039	U18331
	X85759-81	D83661	U43428	U14640
	U18334	U26686	L23806	
	U31511	U16359	L09126	

	U20141	D44591	M92649	
	U05810	X76881	M84373	
	X73029	U02534		
	L24553	L12562		
<b>Endothelial (type III)</b>	X76303-16	U18336		M89952
	L26914	U28933		L27056
	L23210			M95674
	L10693			M99057
	M95296			M89952
	M93718			

Each of these sequences are expressly incorporated herein by reference.

In blood vessels, the endothelial NOS isoform mediates endothelium-dependent vasodilation in response to acetylcholine, bradykinin, and other mediators. Nitric oxide also maintains basal vascular tone and regulates regional blood flow. Nitric oxide levels increase in response to shear stress, i.e., forces on the blood vessels in the direction of blood flow, and to mediators of inflammation. Furchgott & van Houtte (1989); Ignarro (1989).

In the immune system, the macrophage isoform is produced by activated macrophages and neutrophils as a cytotoxic agent. Nitric oxide produced in these cells targets tumor cells and pathogens. Hibbs *et al.* (1988); Nathan (1992); and Marletta (1989).

In the nervous system, the neuronal NOS isoform is localized to discrete populations of neurons in the cerebellum, olfactory bulb, hippocampus, cortex, striatum, basal forebrain, and brain stem. Bredt *et al.* (1990). NOS is also concentrated in the posterior pituitary gland, in the superoptic and paraventricular hypothalamic nuclei, and in discrete ganglion cells of the adrenal medulla. *Id.* The widespread cellular localization of neuronal NOS and the short half-life and

diffusion properties of nitric oxide suggest that it plays a role in nervous system morphogenesis and synaptic plasticity.

During development, NO may influence activity-dependent synaptic pruning, apoptosis, and the establishment of the columnar organization of the cortex. Gally *et al.* (1990), Edelman & Gally (1992). Two forms of long-term synaptic modulation, long-term depression of the cerebellum, Shibuki & Okada (1991), and long-term potentiation (LTP) in the hippocampus, are sensitive to inhibitors of NOS. Bohme *et al.* (1991); Haley *et al.* (1992); O'Dell *et al.* (1991); Schuman & Madison (1991). Thus, nitric oxide may serve as a retrograde neurotransmitter to enhance synaptic function due to correlated firing of pre-and postsynaptic cells.

In the peripheral nervous system, nitric oxide mediates relaxation of smooth muscle. Smooth muscle relaxation in the gut, important to adaptation to a bolus of food and peristalsis, depends upon inhibitory non adrenergic, noncholinergic nerves that mediate their effects via nitric oxide. Boeckstaens *et al.* (1991); Bult *et al.* (1990); Desai *et al.* (1991); Gillespie *et al.* (1989); Gibson *et al.* (1990); Ramagopal & Leighton (1989); Tottrup *et al.* (1991). NOS-containing neurons also innervate the corpus carvornosa of the penis, Burnett *et al.* (1992); Rajfer *et al.* (1992), and the adventitial layer of cerebral blood vessels. Nozaki *et al.* (1993); Toda & Okamura (1990). Stimulation of these nerves can lead to penile erection and dilation of cerebral arteries, respectively. These effects are blocked by inhibition of NOS.

The three isoforms of nitric oxide synthase, the neuronal isoform, the endothelial isoform, and the macrophage isoform, are named after the cell types in which they were first found. However, expression of these isoforms overlaps considerably, and many tissues contain more than one isoform.

Thus, the major roles of nitric oxide include:

- (1) vasodilation or vasoconstriction with resulting change in blood pressure and blood flow;

(2) neurotransmission in the central and peripheral nervous system, including mediation of signals for normal gastrointestinal motility; and

(3) defense against pathogens like bacteria, fungus, and parasites due to the toxicity of high levels of NO to pathogenic organisms.

5 The biological role of NO are described by Schmidt & Walter (1994); Nathan & Xie (1994); and Snyder (1995). NOS genes have been sequenced from different species, e.g., mouse, rat, human, and bovine. These sequences are available on Genbank, *see supra*.

10 Over the last several years, transgenic animals containing specific genetic defects, e.g., resulting in the development of, or predisposition to, various disease states, have been made. These transgenic animals can be useful in characterizing the effect of such a defect on the organism as a whole, and developing pharmacological treatments for these defects.

15 The relevant techniques whereby foreign DNA sequences can be introduced into the mammalian germ line have been developed in mice. *See Manipulating the Mouse Embryo* (Hogan *et al.*, eds., 2d ed., Cold Spring Harbor Press, 1994) (ISBN 0-87969-384-3). At present, one route of introducing foreign DNA into a germ line entails the direct microinjection of a few hundred linear DNA molecules into a pronucleus of a fertilized one-cell egg. Microinjected eggs  
20 may then subsequently be transferred into the oviducts of pseudo-pregnant foster mothers and allowed to develop. It has been reported by Brinster *et al.* (1985), that about 25% of the mice that develop inherit one or more copies of the micro-injected DNA.

25 In addition to transgenic mice, other transgenic animals have been made. For example, transgenic domestic livestock have also been made, such as pigs, sheep, and cattle.

30 Once integrated into the germ line, the foreign DNA may be expressed in the tissue of choice at high levels to produce a functional protein. The resulting animal exhibits the desired phenotypic property resulting from the production of the functional protein.

In light of the various biological functions of nitric oxide, there exists a need in the art to develop transgenic animals, e.g., transgenic mice, wherein the endothelial nitric oxide synthase gene has been modified. There also exists a need in the art to develop methods to test compounds for activity against various pathological states associated with the absence of NOS using these transgenic animals. A further need in the art is to develop treatments for various pathological states using nitric oxide or nitric oxide prodrugs.

### *Summary of the Invention*

This invention satisfies these needs in the art by providing a transgenic non-human animal comprising a disrupted endothelial nitric oxide synthase gene. In embodiments of this invention, the transgenic non-human animal exhibits hypertension or exhibits wound-healing abnormalities. In a specific embodiment of the invention, the transgenic non-human animal is a mouse. Moreover, in specific embodiments of this invention, the endothelial nitric oxide synthase gene is disrupted at exons encoding the NADPH ribose and adenine binding sites.

In other embodiments, this invention provides a method of testing compounds for antihypertensive activity by providing a transgenic non-human animal having a disrupted endothelial nitric oxide synthase gene, wherein the animal exhibits hypertension, administering a compound to be tested to the transgenic animal, and determining the effect of the compound on the blood pressure of the animal.

In a further embodiment, this invention provides a method of testing compounds for wound-healing activity by providing a transgenic non-human animal having a disrupted endothelial nitric oxide synthase gene, administering a compound to be tested to the transgenic animal, and determining the effect of the compound on the wound-healing capabilities of the animal.

In another embodiment, this invention provides a method of treating wounds by applying nitric oxide or a prodrug thereof. In a specific embodiment



thereof, compounds which release nitric oxide into wounds to improve or speed up wound healing.

In an additional embodiment, this invention provides a method of making a transgenic non-human animal of the invention comprising providing an embryonic stem cell comprising an intact eNOS gene; providing a targeting vector capable of disrupting said eNOS gene upon homologous recombination; introducing said targeting vector into said cells under conditions where the intact eNOS gene of said cell and said targeting vector undergo homologous recombination to produce a disrupted eNOS gene; introducing said cells into a blastocyst; implanting the blastocyst into the uterus of a pseudopregnant female; and delivering transgenic animals of the invention from said pseudopregnant female. In order to obtain homozygous mutant mice of the invention, the resulting animals can be bred, and homozygous mutant mice selected.

### ***Brief Description of the Figures***

Figure 1 depicts the targeted disruption of the endothelial NOS gene. This Figure shows restriction maps of the native mouse endothelial NOS gene, the targeting vector, and the disrupted eNOS gene. The targeted vector contains 5' and 3' flanking regions of homology and it is designed to replace the *HindIII-SaII* fragment of the eNOS gene containing exons encoding the NADPH ribose and adenine binding sites (amino acids 1010-1144). NEO refers to the neomycin antibiotic resistance gene. The location of the KS probe, used for Southern blot analysis, is also shown.

Figure 2 shows the results of a Southern blot analysis of genomic DNA isolated from mutant mouse tails, digested with *SpeI* and hybridized to the KS probe, shown in Figure 1. Lanes 1, 2, and 3 show wild-type, heterozygous, and homozygous eNOS mutant mice, respectively. The positions of fragments hybridizing to the wild-type and the disrupted eNOS gene, as depicted in Figure 1, are indicated.

Figure 3 depicts a Western blot analysis of eNOS mutant mice. Brain, heart, lung and aorta samples of wild-type (wt) and mutant (m) mice were tested for immunological reactivity against a mouse monoclonal antibody directed against eNOS. The position of eNOS is shown.

5 Figure 4 depicts the endothelium-dependent relaxation of aortic rings in response to acetylcholine. Panel a shows the effect on wild-type aortic segments. Panel b depicts treatment of wild-type vessel rings with L-nitroarginine (L-NA), a NOS inhibitor. Panel c depicts the endothelium-dependent relaxation of eNOS mutant aortic segments.

10 Figure 5 depicts blood pressure responses to L-NA. Urethane-anesthetized wild-type (solid line) and eNOS mutant mice (dotted line) were measured before and after L-NA administration at time 0.

15 Figure 6 depicts wound healing in eNOS mutant mice. Figure 6A is a micrograph of a 24 hr. wound in a wild-type mouse. Figure 6B is a micrograph of a 5 day wound in a wild-type mouse. Figure 6C is a 24 hr. wound of an eNOS mutant mouse. Figure 6D is a 5 day wound of an eNOS mutant mouse. (ep) refers to the epidermis, while (d) refers to the dermis. Capillaries are indicated by (cap).

### *Detailed Description of Preferred Embodiments*

20 In the description that follows, a variety of various technical terms are used. Unless the context indicates otherwise, these terms shall have their ordinary well-recognized meaning in the art. In order to provide a clearer and more consistent understanding of the specification and claims, the following definitions are provided.

25 **Transgenic.** As used herein, a "transgenic organism" is an organism containing a defined change to its germ line, wherein the change is not ordinarily found in wild-type organisms. This change can be passed on to the organism's progeny. The change to the organism's germ line can be an insertion, a

substitution, or a deletion. Thus, the term "transgenic" encompasses organisms where a gene has been eliminated or disrupted so as to result in the elimination of a phenotype associated with the disrupted gene ("knock-out animals"). The term "transgenic" also encompasses organisms containing modifications to their existing genes and organisms modified to contain exogenous genes introduced into their germ line.

**Nitric oxide synthase (NOS).** As used herein, nitric oxide synthase is an enzyme able to catalyze the formation of nitric oxide. For example, NOS can catalyze the formation of nitric oxide from the terminal guanidino nitrogen of arginine, with the stoichiometric production of citrulline.

**Disrupted gene.** As used herein, "disrupted gene" refers to a gene containing an insertion, substitution, or deletion resulting in the loss of substantially all of the biological activity associated with the gene. For example, a disrupted NOS gene would be unable to express a protein having substantial NOS enzymatic activity.

**Vector.** As used herein, a "vector" is a plasmid, phage, or other DNA sequence, which provides an appropriate nucleic acid environment for a transfer of a gene of interest into a host cell. The cloning vectors of this invention will ordinarily replicate autonomously in eukaryotic hosts. The cloning vector may be further characterized in terms of endonuclease restriction sites where the vector may be cut in a determinable fashion. The vector may also comprise a marker suitable for use in identifying cells transformed with the cloning vector. For example, markers can be antibiotic resistance genes.

**Expression vector.** As used herein, an "expression vector" is a vector comprising a structural gene operably linked to an expression control sequence so that the structural gene can be expressed when the expression vector is transformed into an appropriate host cell.

**Targeting vector.** As used herein "a targeting vector" is a vector comprising sequences that can be inserted into a gene to be disrupted, e.g., by

homologous recombination. Therefore, a targeting vector may contain sequences homologous to the gene to be disrupted.

This invention relates to non-human transgenic animals comprising a disrupted endothelial NOS gene.

5 In order to obtain a transgenic animal comprising a disrupted eNOS gene, a targeting vector is used. The targeting vector will generally have a 5' flanking region and a 3' flanking region homologous to segments of the eNOS gene surrounding an unrelated DNA sequence to be inserted into the eNOS gene. For example, the unrelated DNA sequence can encode a selectable marker, such as  
10 an antibiotic resistance gene. Specific examples of a suitable selectable marker include the neomycin resistance gene (NEO) and the hygromycin  $\beta$ -phosphotransferase. The 5' flanking region and the 3' flanking region are homologous to regions within the eNOS gene surrounding the portion of the gene to be replaced with the unrelated DNA sequence. DNA comprising the targeting  
15 vector and the native eNOS gene are brought together under conditions where homologous recombination is favored. For example, the targeting vector and native eNOS gene sequence can be used to transform embryonic stem (ES) cells, where they can subsequently undergo homologous recombination. For example, J1 embryonic stem cells obtained from Dr. En Li of the Cardiovascular Research  
20 Center of the Massachusetts General Hospital and Dr. Rudolph Jaenisch of the Whitehead Institute of MIT. The targeting vector, pPNT-ENOS, has been deposited with the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Drive, Rockville, Maryland 20852 USA, under the terms of the Budapest Treaty under accession number A.T.C.C. 97469 on March 13, 1996.

25 Proper homologous recombination can be tested by Southern blot analysis of restriction endonuclease digested DNA using a probe to a non-disrupted region of the eNOS gene. For example, the KS probe, identified in Figure 1, can be used. Since the native eNOS gene will exhibit a different restriction pattern from the disrupted eNOS gene, the presence of a disrupted eNOS gene can be  
30 determined from the size of the restriction fragments that hybridize to the probe.

In one method of producing the transgenic animals, transformed ES cells containing a disrupted eNOS gene having undergone homologous recombination, are introduced into a normal blastocyst. The blastocyst is then transferred into the uterus of a pseudo-pregnant foster mother. Pseudo-pregnant foster mothers had been mated with vasectomized males, so that they are in the proper stage of their estrus cycle and their uterus is hormonally primed to accept an embryo. The foster mother delivers a transgenic animal containing a disrupted eNOS gene. Homozygous mutant animals are normally obtained by breeding the transgenic animals.

The extent of the contribution of the ES cells, containing the disrupted eNOS gene, to the somatic tissues of the transgenic mouse can be determined visually by choosing strains of mice for the source of the ES cells and blastocyst that have different coat colors.

The resulting homozygous eNOS mutant animals generated by homologous recombination are viable, fertile, and indistinguishable from wild-type and heterozygous littermates in overall appearance (except for the presence of a selectable marker) or routine behavior. However, the mutant animals can also be sterilized using methods well known in the art, e.g. vasectomy or tubal ligation. *See Manipulating the Mouse Embryo, supra.* The mutant animals can be mated to obtain homozygous or heterozygous progeny. These mutant animals contain substantially less immunoreactive eNOS than wild-type animals. Most preferably, these mutant mice contain no immunoreactive eNOS protein as measured by western blot analysis of brain, heart, lung, or aorta tissue.

A targeting vector such as pPNT-ENOS can be used to create cell lines or primary cell cultures that do not express NOS. The endogenous eNOS gene can be disrupted by introducing the targeting vector into cells containing the eNOS gene to be disrupted and allowing the targeting vector and the endogenous gene to undergo homologous recombination. For example, the targeting vector can be introduced into the cells by electroporation. If both copies of the eNOS gene are to be disrupted, higher concentrations of the selection agent, e.g.,

neomycin or its analog G418 are used. Suitable cell lines and cultures include tumor cells, endothelial cells, epithelial cells, leukocytes, neural cells, glial cells, and muscle cells.

5 The eNOS mutant animals of the invention can be any non-human mammal. In embodiments of this invention, the animal are mice, rats, guinea pigs, rabbits, and dogs. In an especially preferred embodiment of the invention, the eNOS mutant animal is a mouse.

10 These homozygous eNOS mutant animals also exhibit significantly reduced calcium-dependent membrane-associated NOS enzymatic activity. In preferred embodiments of this invention the enzymatic activity in aorta samples is less than  $1.0 \text{ pmol mg}^{-1} \text{ min}^{-1} {}^3\text{H}$ -arginine to citrulline conversion. In most preferred embodiments of this invention the enzymatic activity in aorta samples is less than  $0.5 \text{ pmol mg}^{-1} \text{ min}^{-1} {}^3\text{H}$ -arginine to citrulline conversion. Any residual NOS activity may be due to the presence of neuronal NOS (nNOS) in neurons in  
15 the perivascular plexus, and not to the expression of any residual nondisrupted eNOS genes.

Endothelium-derived relaxing factor is absent or significantly reduced in eNOS mutant animals. However, vascular smooth-muscle responses are intact. For example, the aortic rings of mutant mice show no relaxation to acetylcholine,  
20 while aortic rings from wild-type mice manifest a dose-dependent relaxation to acetylcholine. See Figure 4. Moreover, treatment of wild-type aortic rings with  $10^{-4}\text{M}$  L-nitroarginine has no effect on vascular tone by itself, but blocks the relaxation in response to acetylcholine. *Id.* Treatment of the eNOS mutant aortic rings with  $10^{-4}\text{M}$  L-nitroarginine also has little or no effect on vessel tone either  
25 by itself or on the response to acetylcholine. The maximum dilation of norepinephrine pre-contracted rings from eNOS mutant mice to sodium nitroprusside is similar to wild-type mice. This indicates that vascular smooth-muscle responses in eNOS mutant animals are intact.

30 The eNOS mutant animals exhibit a significantly higher blood pressure than the wild-type animals. In specific embodiments of this invention cNOS

mutant mice exhibit a mean blood pressure significantly higher than 81 mm Hg. In a preferred embodiment of this invention, eNOS mutant mice exhibit a mean blood pressure greater than about 100 mm Hg. In a most preferred embodiment of this invention, eNOS mutant mice exhibit a mean blood pressure of about 110 mm Hg. Analogous increases in mean blood pressure are expected in other non-murine eNOS mutant (knock-out) animals.

L-NA and other NOS inhibitors cause a rise in blood pressure in many species including humans, rats, guinea pigs, rabbits, dogs, and mice. This effect is consistent with a role of basal nitric oxide production in vasodilation, because inhibition of eNOS would lead to less basal vasodilation and result in hypertension. However, eNOS mutant mice show a decrease in blood pressure in response to L-NA. See Figure 5. This hypotensive effect is blocked by L-arginine and is not observed with D-nitroarginine. This suggests that pharmacological blockers may have effects in addition to NOS inhibitors, or that non-endothelial NOS isoforms are involved in the maintenance of blood pressure. For example, nNOS is present both in vasomotor centers of the central nervous system and in perivascular nerves. However, effects of its blockade suggest that it plays a vasodilatory role. Mutant neuronal NOS mice have blood pressures similar to wild-type mice, but they have a tendency towards hypotension when exposed to anaesthesia, which is consistent with a possible role for nNOS in maintaining, not reducing, blood pressure. Multiple roles for endothelial and non-endothelial NOS isoforms in vasodilation and vasoconstriction may explain the observed variability in maximal pressor effects of various NOS inhibitors.

There is evidence that in hypertension the amount of NO produced by the endothelium decreases in humans. The eNOS knockout mice mimic this effect, since their endothelium also does not produce any NO. Consequently, these mice serve as a useful model for hypertension. Thus, establishing an anti-hypertensive effect in eNOS mutant animals of the invention for a compound other than an NOS inhibitor, such as a compound that induces the production of NO, would be predictive that this compound would have anti-hypertensive properties in other

animals, including humans. However, the opposite result would be expected for a compound that inhibits NOS.

5 An inhibitor of NOS would be expected to raise blood pressure in wild-type animals, but stabilize or reduce blood pressure in eNOS mutant animals. In eNOS mutant animals, an NOS inhibitor would inhibit the residual NOS, mostly nNOS. nNOS mutant animals may have a tendency toward hypotension. Thus, the preferential inhibition of nNOS in the mutant animals should result in the same or lower blood pressure. However, in normal animals, the role of eNOS on blood pressure regulation is much more pronounced than the role of nNOS. Therefore, the predominant effect of an NOS inhibitor in normal animals would be to inhibit eNOS, which raises blood pressure. This is demonstrated in Example 5, *infra*.

10 In addition, there is also evidence that in atherosclerosis, diabetes and normal aging, the amount of NO produced by the endothelium decreases in humans. Thus, these mice would serve as a useful model not just for hypertension, but also for vascular responses in atherosclerosis, diabetes and normal aging.

15 The eNOS mutant animals of this invention are useful as an animal model to study hypertension. For example, various compounds could be tested for an anti-hypertensive effect in the eNOS mutant animals. Specifically, compounds that are not NOS inhibitors can be tested. In more preferred embodiments, compounds that are not eNOS inhibitors are tested. In other preferred embodiments compounds that induce NO production are tested. In additional preferred embodiments, compounds that induce NO production in the endothelium are tested. Therefore, the invention provides methods for screening compounds using the eNOS mutant animals as an animal model to identify compounds useful in treating hypertension. This aspect of the invention is useful to screen compounds from a variety of sources. Examples of compounds that can be screened using the method of the invention include but are not limited to

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rationally designed and synthetic molecules, plant extracts, animal extracts,



inorganic compounds, mixtures, and solutions, as well as homogeneous molecular or elemental samples.

The invention, therefore, provides a method of screening compounds, comprising: providing a transgenic non-human animal having a disrupted eNOS gene and exhibiting hypertension, administering a compound to be tested to the transgenic animal; determining the effect of the compound on the blood pressure of said animal; and correlating the effect of the compound on the blood pressure of the animal with an anti-hypertensive effect of said compound.

The compounds to be tested can be administered to the transgenic non-human animal having a disrupted eNOS gene in a variety of ways well known to one of ordinary skill in the art. For example, the compound can be administered by parenteral injection, such as subcutaneous, intramuscular, or intra-abdominal injection, infusion, ingestion, suppository administration, and skin-patch application. Moreover, the compound can be provided in a pharmaceutically acceptable carrier. See Remington's Pharmaceutical Sciences (1990). The effect of the compound on blood pressure can be determined using methods well known to one of ordinary skill in the art.

In addition, the eNOS mutant animals of this invention unexpectedly exhibit abnormal wound-healing properties. For example, these animals often develop spontaneous wounds that do not heal. In contrast to normal mice, who healed their wounds within 5 days, the eNOS mutant mice exhibit significantly different wound healing properties. First, the eNOS mutant mice exhibit spontaneous wounds that never heal. Second, in experiments where wounds were created, the eNOS mutant mice heal more slowly than normal animals. Healing of eNOS mutant mice typically takes 2-3 times as long as normal mice. The exact healing time will depend on the type of wound inflicted. Moreover, two specific features of normal wound healing are abnormal in the eNOS mutant mice:

- 1) growth of the epithelial layer of skin across the wound to bridge the gap and close the wound; and

2) neovascularization in the granulation tissue that fills the wound.

In the eNOS mutant mice, migration of epithelial cells to the site of the wound is delayed, with epithelial cells remaining at the edge of the wound after five days. The connective tissue is also markedly abnormal, containing few or no new blood vessels. The results demonstrate an important role for eNOS in angiogenesis and epithelial cell migration during wound healing.

The eNOS mutant animals of this invention are useful as an animal model to study wound healing. For example, various compounds could be tested for a wound healing effect in the eNOS mutant animals. Therefore, the invention provides methods for screening compounds using the eNOS mutant animals as an animal model to identify compounds useful in enhancing wound healing. This aspect of the invention is useful to screen compounds from a variety of sources. Examples of compounds that can be screened using the method of the invention include but are not limited to rationally designed and synthetic molecules, plant extracts, animal extracts, inorganic compounds, mixtures, and solutions, as well as homogeneous molecular or elemental samples. For example, various compounds designed to improve wound healing can be tested. For example, compounds that deliver NO to the healing wound can be used. Establishing an enhancement of wound healing by a compound in eNOS mutant animals is predictive that this compound would enhance wound healing in other animals, including humans.

The invention, therefore, provides a method of screening compounds, comprising: providing a transgenic non-human animal having a disrupted eNOS gene and exhibiting abnormal wound healing properties; administering a compound to be tested to the transgenic animal; determining the effect of the compound on the wound healing properties of said animal; and correlating the effect of the compound on the wound healing properties of the animal with a wound healing effect of said compound.

The compounds to be tested can be administered to the transgenic non-human animal having a disrupted eNOS gene in a variety of ways well known to one of ordinary skill in the art. For example, the compound can be administered by parenteral injection, such as subcutaneous, intramuscular, or intra-abdominal injection, infusion, ingestion, suppository administration, and skin-patch application. Moreover, the compound can be provided in a pharmaceutically acceptable carrier. See Remington's Pharmaceutical Sciences (1990).

Thus, the eNOS mutant animals of this invention are also useful as animal models to study wound healing.

Moreover, since the synthesis of nitric oxide appears to enhance wound healing, prodrugs that release nitric oxide *in situ* may improve or speed up healing. Examples of suitable compounds that release nitric oxide include nitroglycerin, sodium nitroprusside, and SIN-1. The extent to which these and other similar compounds improve or speed up wound healing can be determined experimentally. For example, the compound could be applied at the wound to a patient in need of treatment and its effect on wound healing can be quantified.

### *Examples*

#### *Example 1*

##### *Targeted Disruption of the Endothelial NOS Gene*

The endothelial NOS gene was cloned by screening a mouse genomic library, obtained from Stratagene, using a human eNOS cDNA clone, obtained from Kenneth D. Bloch, as described in Janssens *et al.* (1992), Genbank accession number M93718. The targeting vector was derived from the pPNT vector, which contains thymidine kinase gene and the neomycin resistance gene. See Figure 1. Tybulewicz *et al.* (1991). The targeting vector contains 5' and 3' flanking regions of homology to the eNOS gene, and is designed to replace the

*HindIII-SalI* fragment that contains exons encoding the NADPH ribose and adenine binding sites of the eNOS protein (amino acids 1010-1144) following homologous recombination.

5 J1 ES cells were grown as described in Li *et al.* (1992) on irradiated embryonic fibroblast feeder cells in media containing 200 units/ml leukocyte inhibitory factor. For electroporation,  $10^7$  cells were mixed with targeting vector DNA at 150  $\mu$ g/ml. A Bio-Rad gene pulsar was used to electroporate the DNA into the cells at a setting of 960  $\mu$ F capacitance, 250 mV. The targeting vector and the native eNOS gene were then able to undergo homologous recombination.  
10 The cells were plated on neomycin-resistant irradiated fibroblast feeder cells, and selection with 150  $\mu$ g/ml G418 and 2  $\mu$ M FIAU was started 48 hours later. Doubly resistant colonies were picked seven days after electroporation and grown in 24-well plates.

## Example 2

### 15 *Generation of Chimeric Mice with Germline Transmission*

Blastocysts were isolated from C57 BL/6 mice on day 3.5 of pregnancy and 20-25 ES cells, following homologous recombination and selection, were injected into the uterine horn of pseudo-pregnant (C57 BL/6 x DBA/2) F1 mice. Chimeric mice were identified by the agouti contribution of the ES cells to the coat color, and were back-crossed to C57 BL/6 mice. Germline transmission was  
20 determined by the presence of agouti mice in the offspring.

Proper recombination was demonstrated by Southern blot analysis of *SpeI* digested genomic DNA using the KS probe shown in Figure 1. Back-crossed mice were screened by Southern blot, and heterologous mice were selected. In  
25 Figure 1, lanes 1, 2 and 3 show wild-type, heterozygous, and homozygous mutant mice, respectively. The positions of the hybridizing fragments for the wild-type and the disrupted eNOS gene are shown. The results demonstrate that the

targeted disruption of the eNOS gene is present in the germline of the transgenic mice.

Western blot analysis of tissue samples from eNOS mutant mice was also performed. 10  $\mu$ g protein extracts from the brain, heart, lung, and aorta/vena cava of wild-type and eNOS mutant animals were electrophoresed through a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The blot was incubated with mouse monoclonal antibodies directed against endothelial NOS (Transduction Research Laboratories), and specific hybridization was localized using chemiluminescent alkaline phosphatase conjugated with anti-mouse antibody (ECL, Amersham). Hybridization of endothelial NOS is observed for the wild-type samples, but not in the eNOS mutant samples. The heart, lung, and aorta samples show a band at relative molecular mass of 50 kDa in both wild-type and eNOS mutant samples, which represents mouse immunoglobulin heavy chains present in the tissue samples. The antibody used was directed against a peptide corresponding to amino acids 1030-1209, a region which overlaps with the region deleted in the eNOS mutant mice. See Figure 3. This demonstrates that the eNOS mutant mice (homozygous) produce no immunoreactive undisturbed eNOS, further demonstrating successful transmission of the disruption to the germline of the transgenic mice.

Aorta samples from eNOS mutant mice were tested for calcium-dependent membrane-associated NOS enzymatic activity. This enzymatic activity was reduced to about 0.5 pmol  $\text{mg}^{-1} \text{min}^{-1}$ . The residual activity is likely due to neuronal NOS in neurons in the perivascular plexus.

### ***Example 3***

#### ***Determination of blood pressure of eNOS mutant and wild-type mice***

Mice were kept at normal temperature (37°C), anesthetized with urethane (1.5 mg/kg, intraperitoneal injection) or halothane inhalation, and ventilated using

an SAR-830 mouse ventilator (CWE Instruments). Depth of anesthesia was adjusted to keep the blood pressure of animals unresponsive to tail-pinch with forceps. End-tidal CO<sub>2</sub> was monitored with a microcapnometer and kept constant by adjustment of respiratory parameters. The right femoral artery was cannulated using stretched PE-10 polyethylene tubing (Clay Adams) for mean arterial blood pressure recordings using an ETH-400 transducer and a MacLab data acquisition system (ADI Instruments). For awake measurements, the femoral artery catheter was placed under halothane anesthesia and the wound was covered with 1% xylocane ointment to diminish discomfort. Recordings were made within one hour of the procedure. Data are expressed as means with standard deviation. Statistical evaluation was performed by t-test.

TABLE I Blood pressure of eNOS mutant and wild-type mice

	Wild-type mice			eNOS mutant mice		
	Urethane	Halothane	Awake	Urethane	Halothane	Awake
Mean BP (mmHG)	81	90	97	110*	109*	117*
s.d.	9	12	8	8	11	10
<i>n</i>	16	15	14	17	18	17

\*P<0.01 for eNOS mutant animals vs. wild-type mice.

There is no statistically significant difference between the blood pressure of nNOS mutant mice and wild-type mice using this procedure.

Similar results are obtained with different methods of anesthesia and in the awake state. Blood pressure is the same for the wild-type SV129 strain, wild-type C57 B16 strain, and littermates of the eNOS mutant animals that are wild-type at the eNOS locus.

#### Example 4

##### *Endothelium-dependent Relaxation of Aortic Rings in Response to Acetylcholine from eNOS Mutant and Wild-type Mice*

5 The thoracic aorta was dissected from wild-type and eNOS mutant mice and placed in physiological saline aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. 4 mm segments of the aorta were mounted on tungsten wires in conventional myographs and maintained at optimal tension in physiological saline for 1 hour at 37°C. The rings were pre-contracted with 10<sup>-7</sup> M norepinephrine and exposed to increasing concentrations of acetylcholine (ACh) from 10<sup>-8</sup> M to 3 x 10<sup>-5</sup> M. 2-3 segments  
10 were collected from each mouse, and five mice were used from the wild-type and eNOS mutant groups. Mean data from each animal were used. Respective tracings are shown. Acetylcholine concentrations are expressed in logM.

Figure 4A depicts wild-type aortic segments responding to acetylcholine with dose-dependent relaxation. At 3 x 10<sup>-5</sup> M, 60.3 ± 14.6% of pre-addition tone was present. Figure 4B depicts treatment of the wild-type vessel rings with 10<sup>-4</sup> M L-NA for 1 hour and shows abolished acetylcholine-induced relaxation. Figure 4C shows that eNOS mutant aortic segments do not relax to acetylcholine, demonstrating that EDRF activity is absent from eNOS mutant mice. L-NA has no additional affect on eNOS vessel segments.

20 Thus, aortic rings from wild-type mice manifest a dose-dependent relaxation to acetylcholine, while aortic rings from eNOS mutant animals show no relaxation to acetylcholine. Treatment of wild-type aortic rings with 10<sup>-4</sup> M L-NA has no effect on vascular tone by itself, but blocks the relaxation in response to acetylcholine. Treatment of the eNOS mutant aortic rings with  
25 10<sup>-4</sup> M L-NA has no effect on vessel tone, either by itself or in response to acetylcholine. The maximum dilation of norepinephrine pre-contracted rings from eNOS mutant mice to sodium nitroprusside is no different from wild-type mice, indicating that vascular smooth-muscle responses are intact.

### Example 5

#### *Blood Pressure Responses to L-NA for Wild-type and eNOS Mutant Mice*

Mean arterial blood pressure (MABP) of urethane-anesthetized wild-type (solid line in Figure 5) and eNOS mutant mice (dotted line in Figure 5) were measured by femoral artery catheterization and recorded for 30 minutes of baseline before L-NA administration (arrows in Figure 5). At time 0, 12 mg/kg of L-NA was given intraperitoneally. Monitoring for 1 hour shows that the blood pressure of wild-type mice rose from a baseline of 78 mm Hg to 109 mm Hg (n = 11). The blood pressure of eNOS mutant mice dropped from a baseline of 98 mm Hg to 66 mm Hg (n = 5). Each mouse in the wild-type group responded to L-NA with a rise in blood pressure, and each mouse in the eNOS mutant group responded with a drop in pressure. Mean arterial blood pressure differences between wild-type and eNOS mutant animals are statistically significant by the t-test (#,  $p < 0.01$ ). Differences between baseline blood pressures and following L-NA treatment were also statistically significant (\*,  $P < 0.05$  by ANOVA followed by Dunnett). These effects were prevented by L-arginine (200 mg/kg, intraperitoneal), and were not seen with D-nitroarginine (12 mg/kg). The heart rate of eNOS mutant mice and wild-type mice did not differ, and L-NA had no effect on heart rate.

These results support the conclusion that eNOS in the endothelium regulates blood pressure. The major NOS isoform in the endothelium is eNOS. However, a small amount of nNOS is also present. Disruption of the eNOS gene raises blood pressure, while disruption of the nNOS gene stabilizes or lowers blood pressure. Thus, inhibition of NOS in wild-type animals by L-NA, which would predominantly inhibit eNOS, would be expected to raise blood pressure. This is shown in Figure 5. However, inhibition of NOS in eNOS mutant mice would not be expected to raise blood pressure since the effect of L-NA inhibition in the mutants would be to inhibit nNOS and not eNOS. Since nNOS maintains



or raises blood pressure levels, *see supra*, inhibition of nNOS would be expected to lower blood pressure levels. This is also seen in Figure 5.

### ***Example 6***

#### ***Screening of Compounds for Anti-hypertensive Effects using eNOS Mutant Mice***

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The eNOS mutant mice exhibit hypertension. Compounds that are associated with NO production in the endothelium, thereby replacing eNOS enzymatic activity, are screened for anti-hypertension activity in eNOS mutant mice. These compounds can be administered to the eNOS mutant mice using pharmaceutically acceptable methods. *See* Remington's Pharmaceutical Sciences (1990). For example, the compound to be screened can be administered at various concentrations by parenteral injection, infusion, ingestion, and other suitable methods in admixture with a pharmaceutically acceptable carrier. The effect of various concentrations of the screened compound on blood pressure is measured relative to control eNOS mutant animals that have not been administered the compound.

A significant decrease in blood pressure of the eNOS mutant mice by a screened compound is indicative that this compound would exhibit beneficial anti-hypertensive properties in other animals and in humans.

20

### ***Example 7***

#### ***Wound Healing in eNOS Mutant Mice***

25

It was observed that eNOS mutant mice, but not wild-type or nNOS mutant mice, tend to develop chronic wounds. Therefore, eNOS mutant mice were tested in a model of wound healing involving a full thickness transverse incision overlying the lumbar area. The incisions penetrated the deep dermis

down to the skeletal muscle. In Figure 6, the histological appearance of these wounds at 24 hours and at 5 days following incision, for wild-type and eNOS mutant mice, are shown. At 24 hours, the wounds are very similar. The epidermis (ep) and the deep dermis (d) are transected. Inflammatory cells are seen in both wild-type and eNOS mutant mice.

By five days, wild-type mice have healed. The epithelial layer is reconstituted, and granulation tissue with newly sprouted capillaries fills in the scar. The dermis, which has been cut, remains absent in the healed wound. Thus, normal healing involves two quantifiable features: rapid epithelial migration from the wound edges, and the development of new capillaries. neovasculation, in the connective tissue matrix.

In these eNOS mutant animals, little or no healing has been observed at 5 days. The migration of epithelial cells is delayed, and the epithelial cells remain at the edge of the wound (ep). The connective tissue is markedly abnormal, and contains few or no new vessels. These results demonstrate an important role for eNOS in angiogenesis and epithelial cell migration during wound healing.

### *Example 8*

#### *Screening of Compounds for Wound Healing Affects using eNOS Mutant Mice*

The eNOS mutant mice exhibit spontaneous wounds. Compounds to be screened for wound healing activity can be administered to the eNOS mutant mice in a pharmaceutically acceptable excipient. For example, the compound can be administered at various concentrations to the wound directly as an ointment or salve. Alternatively, other pharmaceutically acceptable modes of administration can be used. For example, a pharmaceutical composition comprising the compound can be administered by parenteral injection, infusion,

ingestion, skin-patch application, and other suitable methods. The effect of the compound is measured relative to control eNOS animals that have not been administered the compound.

5 A significant enhancement of wound healing on the spontaneous wounds of eNOS mutant mice by a screened compound would indicate that this compound exhibits beneficial wound healing properties in other animals and in humans.

Particularly preferred compounds for screening are compounds known to release NO, such as nitroglycerin, sodium nitroprusside, and SIN-1.

10 Similarly, eNOS mutant mice having artificially inflicted wounds can also be used in such a screening assay. For example, the effect of various compounds on a full thickness transverse incision, as described in the preceding example, can be used as a screening assay.

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<sup>1</sup> All of the literature cited in this application is expressly incorporated herein by reference.

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***What Is Claimed Is:***

1. A transgenic non-human animal comprising a disrupted endothelial nitric oxide synthase gene.
- 5 2. The transgenic non-human animal as claimed in claim 1, wherein said animal is a mouse.
3. The transgenic non-human animal as claimed in claim 2, wherein said transgenic animal exhibits hypertension.
- 10 4. The transgenic non-human animal as claimed in claim 2, wherein said animal exhibits wound healing abnormalities.
5. The transgenic non-human animal as claimed in claim 4, wherein said animal develops spontaneous wounds that do not heal.
- 15 6. The transgenic non-human animal as claimed in claim 4, wherein said animal exhibits retarded neovascularization.
7. The transgenic non-human animal as claimed in claim 1, wherein endothelium-derived releasing factor is absent.
- 20 8. The transgenic non-human animal as claimed in claim 1, wherein sequences of the endothelial nitric oxide synthase gene encoding NADPH ribose and adenine binding sites are disrupted.
- 25 9. The transgenic non-human animal as claimed in claim 8, wherein nucleotides encoding amino acids 1010-1144 of said endothelial nitric oxide synthase gene are replaced with a sequence from a targeting vector.

10. The transgenic non-human animal as claimed in claim 9, wherein said sequence from the targeting vector encodes a neomycin resistance gene.

5 11. The progeny of the transgenic non-human animal as claimed in claim 1.

12. A method of screening compounds for anti-hypertensive activity, comprising:

10 (a) providing a transgenic non-human animal having a disrupted endothelial nitric oxide synthase gene and exhibiting hypertension;

(b) administering a compound to be tested to said transgenic animal;

15 (c) determining the effect of said compound on the blood pressure of said animal; and

(d) correlating the effect of said compound on the blood pressure of said animal with an anti-hypertensive effect of said compound;

wherein said compound is not an NOS inhibitor.

20 13. The method of screening compounds as claimed in claim 12, wherein said compound induces NO synthesis in the endothelium.

14. A method of screening compounds for wound-healing activity, comprising

25 (a) providing a transgenic non-human animal having a disrupted endothelial nitric oxide synthase gene and exhibiting abnormal wound-healing properties;

(b) administering a compound to be tested to said transgenic animal;

(c) determining the effect of said compound on the wound-healing properties of said animal; and

(d) correlating the effect of said compound on the wound-healing properties of said animal with a wound-healing effect of said compound.

5

15. A method of treating a wound comprising applying a nitric oxide releasing compound to said wound.

10

16. A method of treating a wound as claimed in claim 15, wherein said nitric oxide releasing compound is selected from the group consisting of nitroglycerin, sodium nitroprusside, and SIN-1.

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17. A method of making a transgenic non-human animal having a disrupted endothelial nitric oxide synthase gene, comprising:

(a) providing an embryonic stem cell comprising an intact eNOS gene;

(b) providing a targeting vector capable of disrupting said eNOS upon homologous recombination;

20

(c) introducing said targeting vector into said embryonic stem cell under conditions where said targeting vector will undergo homologous recombination with the eNOS gene of said stem cell to produce a disrupted eNOS gene;

(d) introducing said embryonic stem cell into a blastocyst;

(e) implanting said blastocyst containing the disrupted eNOS gene into the uterus of a pseudopregnant female; and

25

(f) delivering a transgenic animal comprising a disrupted eNOS gene from said pseudopregnant female.



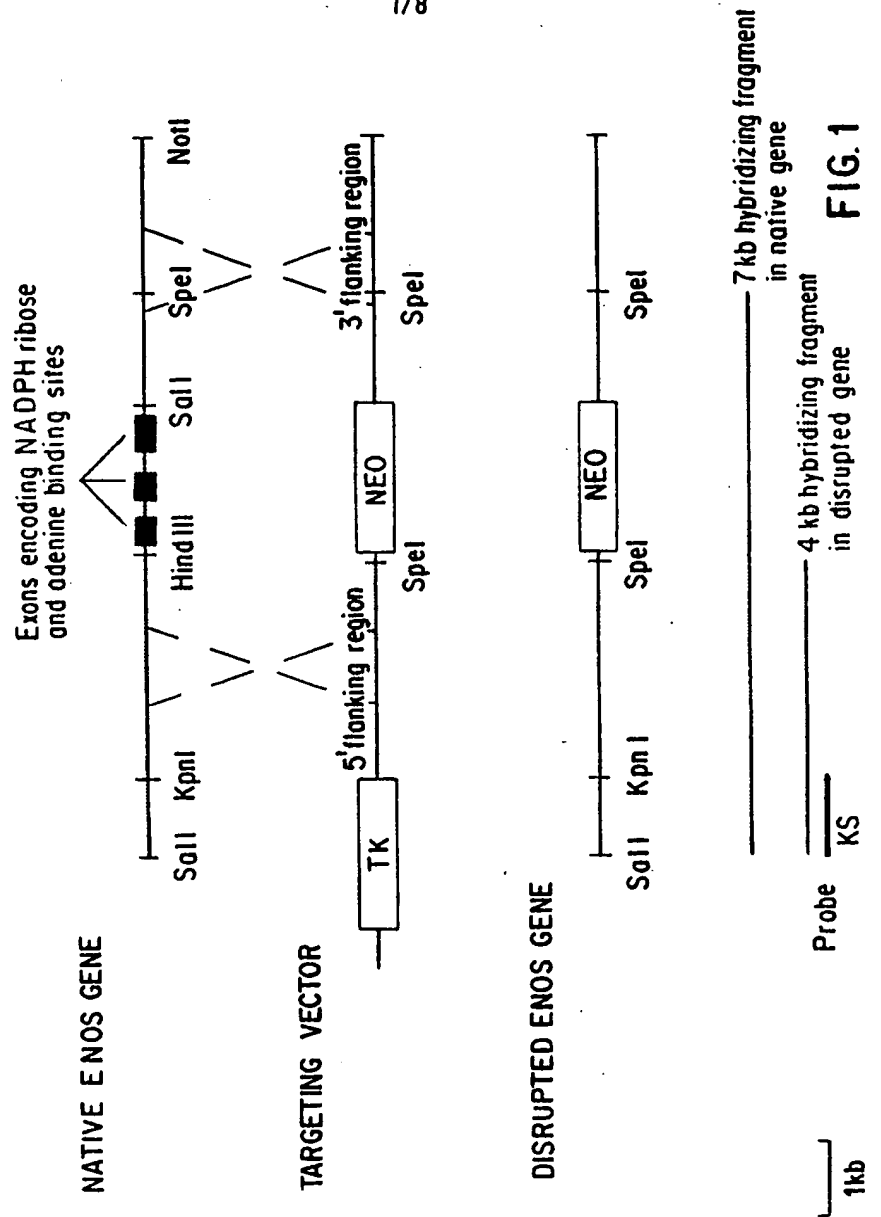
18. The method of making a transgenic non-human animal as claimed in claim 17, wherein said pseudopregnant female and said transgenic animal is a mouse.

5 19. The method of making a transgenic non-human animal as claimed in claim 17, wherein said method further comprises breeding said transgenic animals to obtain homozygous eNOS mutant animals.

20. A cell line comprising a disrupted eNOS gene.

21. The cell line as claimed in claim 20, wherein substantially all cells of said cell line have both copies of said eNOS gene disrupted.

10 22. The cell line as claimed in claim 20, wherein said cell line is selected from the group consisting of a tumor cell line, an endothelial cell line, an epithelial cell line, a leukocyte cell line, a neural cell line, a macrophage cell line, a glial cell line, and a muscle cell line.



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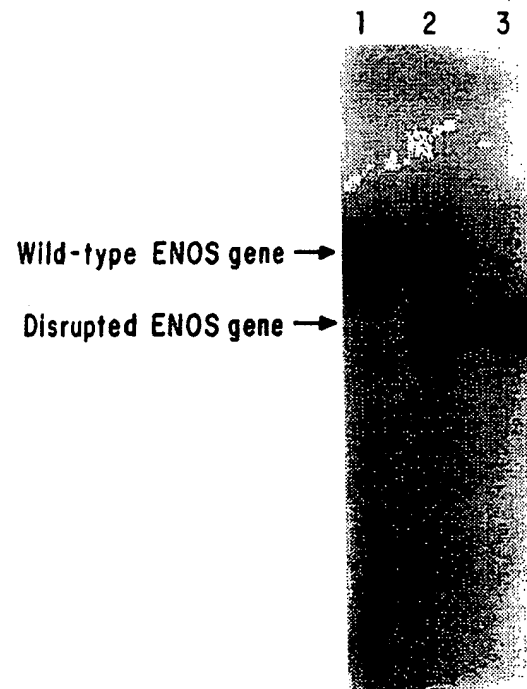


FIG. 2

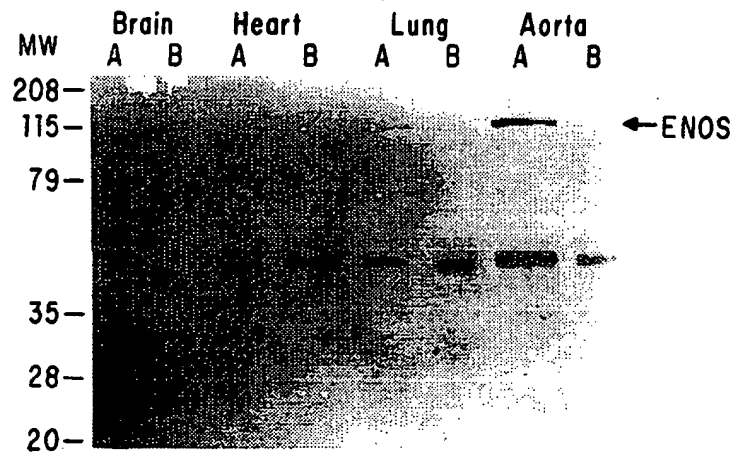
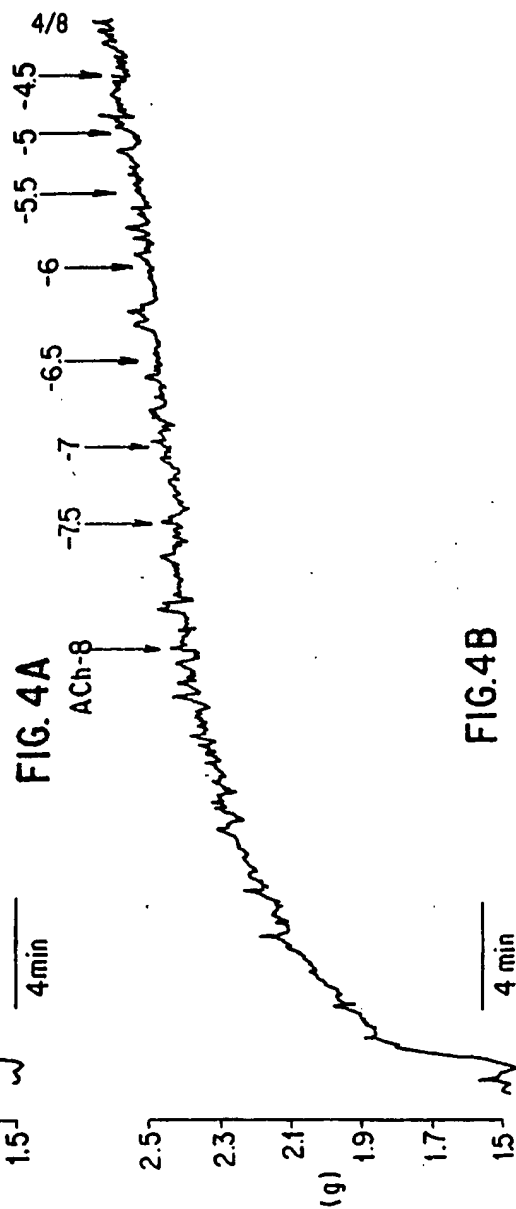
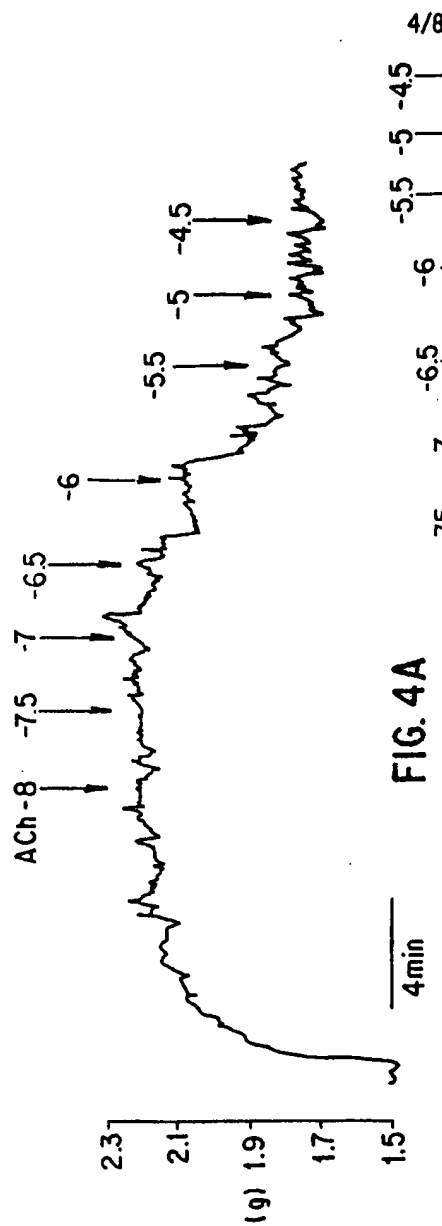


FIG. 3



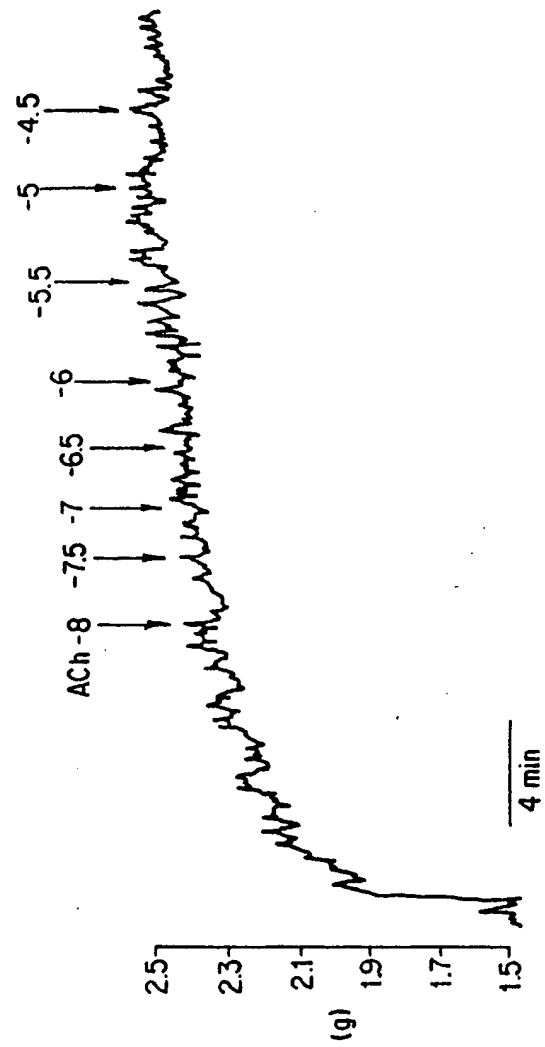


FIG. 4C

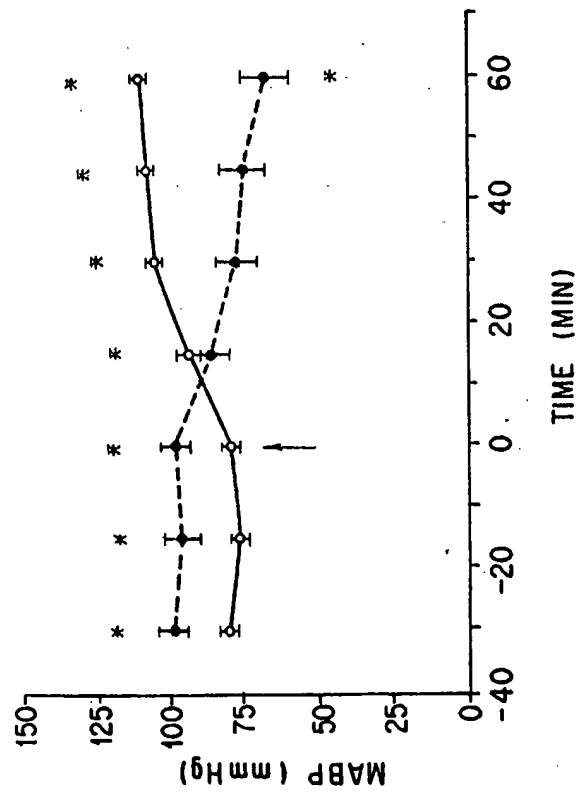


FIG. 5

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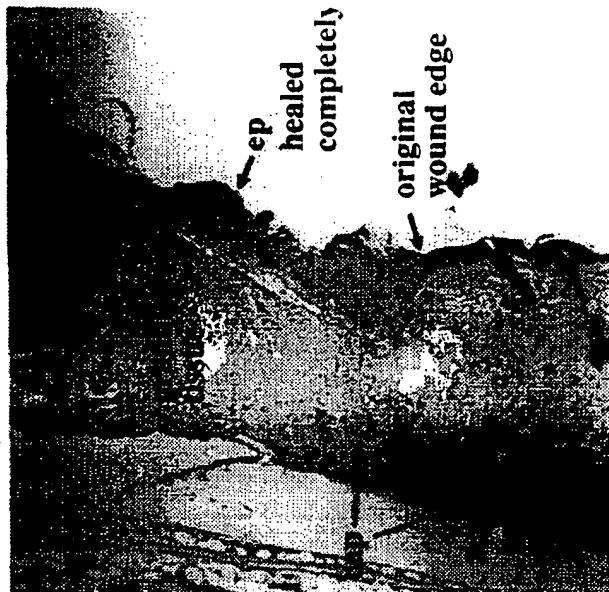


FIG. 6B



FIG. 6A



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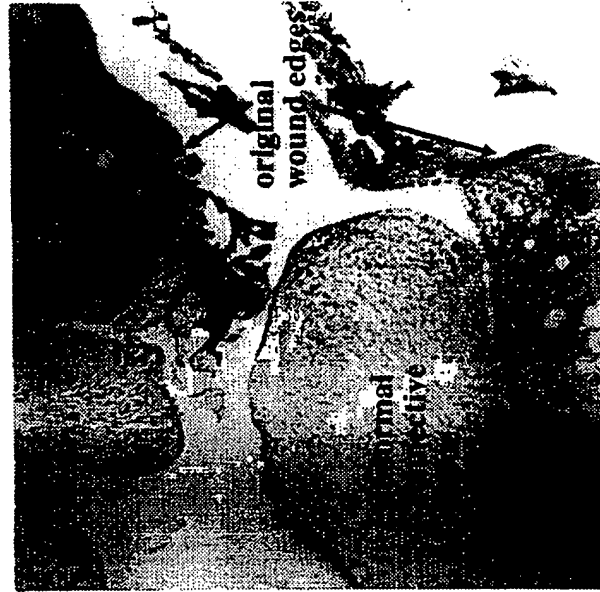


FIG. 6D



FIG. 6C

SUBSTITUTE SHEET (RULE 26)

# INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>10</u> , line <u>line 21</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <b>AMERICAN TYPE CULTURE COLLECTION</b>	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit 13 March 1996	Accession Number A.T.C.C. 97469
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
<p>pPNT-ENOS</p> <p>In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).</p>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
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## INTERNATIONAL SEARCH REPORT

Internat Application No  
PCT/US 96/03467A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N9/02 A01K67/027 C12N5/10 C12N15/90 A61K31/04  
G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A01K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, vol. 377, 21 September 1995, pages 239-243, XP002021109 HUANG, P.L. ET AL.: "Hypertension in mice lacking the gene for endothelial nitric oxide synthase" Figure 1; page 241, left-hand column, lines 15-16 and Figure 3 ---	1-13
A	CELL, vol. 75, no. 7, 31 December 1993, pages 1273-1286, XP000607862 HUANG, R.L. ET AL.: "Targeted Disruption of the neuronal nitric oxide synthase gene" Figure 1 --- -/-	1

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

13 December 1996

Date of mailing of the international search report

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Alt, G

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/03467

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 223, no. 3, August 1994, pages 719-726, XP002021110 MIYAHARA, K. ET AL.: "Cloning and structural characterization of the human endothelial nitric-oxide-synthase gene" Figure 1</p> <p>---</p>	1
A	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 3025.30, 25 October 1992, pages 21277-21280, XP002021111 HECK, D.E. ET AL.: "Epidermal growth factor suppresses nitric oxide and hydrogen peroxide production by keratinocytes" page 21278, right-hand column, first paragraph - page 21279, right-hand column, first paragraph</p> <p>---</p>	14
A	<p>NEUROSCIENCE LETTERS, vol. 200, no. 1, 10 November 1995, pages 17-20, XP000610409 BENRATH, J. ET AL.: "Substance P and nitric oxide mediate wound healing of ultraviolet photodamaged rat skin: evidence for an effect of nitric oxide on keratinocyte proliferation" page 19, right-hand column, second paragraph - page 20, left-hand column, lines 1-6</p> <p>-----</p>	14

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/03467

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

4 inventions \* see continuation-sheet PCT/ISA/210 \*

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

- claims 1-14: Transgenic animal having a disrupted eNOS gene and use of these animals
- claims 15-16: Method of treating a wound by applying a nitric oxide releasing compound
- claims 17-19: Method of making a transgenic animal having a disrupted eNOS gene by using a targetting vector capable of homologous recombination
- claims 20-22: Cell line comprising a disrupted eNOS gene